JOURNAL OF VIROLOGY, Aug. 1985, p. 314–321
0022-538X/85/080314-08$02.00/0

Vol. 55, No. 2

Rous Sarcoma Virus Variants That Encode *src* Proteins with an Altered Carboxy Terminus Are Defective for Cellular Transformation

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The *src* gene of Rous sarcoma virus (v-*src*) and its cellular homolog, the c-*src* gene, share extensive sequence homology. The most notable differences between these genes reside in the region encoding the carboxy terminus of the *src* proteins. We constructed mutations within the 3′ end of the v-*src* gene to determine the significance of this region to the transforming potential of the v-*src* protein, pp60$^{v\text{-}src}$. The mutants CH*dl*300 and CH*is*1511 contain mutations that alter the last 23 amino acids of pp60$^{v\text{-}src}$, whereas the mutant CH*is*1545-C contains a linker insertion that alters the last 11 amino acids of pp60$^{v\text{-}src}$, and the mutant CH*is*1545-H contains a linker insertion that results in a 9-amino-acid insertion at position 415. Plasmids bearing each of these mutations were unable to transform chicken cells when introduced into these cells by DNA transfection. In addition, the structurally altered *src* proteins encoded by the mutants had much-reduced levels of tyrosine protein kinase activity in vivo, as measured by autophosphorylation and phosphorylation of the 34,000-M_r cellular protein, and in vitro, as determined by measuring the level of pp60^{src} autophosphorylation. These data indicate that the carboxy-terminal amino acid sequences play an important role in maintaining the structure of the catalytic domain of pp60$^{v\text{-}src}$. In contrast, the transfection of chicken cells with plasmid DNA containing a chimeric v-c-*src* gene resulted in morphological cell transformation and the synthesis of an enzymatically active hybrid protein. Therefore, the carboxy-terminal sequence alterations observed in the c-*src* protein do not alone serve to alter the functional activity of a hybrid v-c-*src* protein appreciably.

The induction of cellular transformation by Rous sarcoma virus (RSV) requires the functional expression of the viral *src* (v-*src*) gene product pp60^{src} (1, 3, 25). pp60^{src} is a tyrosine protein kinase and is a member of a family of related oncogene products, each of which is either a functional tyrosine protein kinase or is structurally related to the tyrosine kinases (1, 10, 20, 27, 34). In vitro, pp60^{src} catalyzes the tyrosine-specific phosphorylation of a variety of substrates (9, 11, 24), including phosphatidyl inositol lipids (36, 55). In vivo, the cellular expression of functional pp60^{src} results in the tyrosine-specific phosphorylation of a number of cellular proteins (12, 13, 19, 44, 47). The intrinsic kinase activity of pp60^{src}, coupled with the transformation-specific tyrosine phosphorylation of certain cellular proteins and lipids, suggests that the *src* protein plays a critical role in cellular transformation via the phosphorylation of cellular target molecules.

The biochemical characterization of pp60^{src} and structurally altered *src* proteins encoded by RSV mutants has led to the conclusion that pp60^{src} contains several distinguishable functional domains (4, 5, 15, 40). Considerable evidence has shown that pp60^{src} is associated with the plasma membrane via an amino-terminal domain (14, 15, 32) and that this association with the membrane is required for cellular transformation (17). The observation that pp60^{src} contains lipid covalently bound to an amino-terminal residue, glycine (45), suggests that this moiety may contribute to the anchoring of the *src* protein in the plasma membrane (7, 8, 17, 48). In addition, the analysis of deletion mutations within the 5′ half of the *src* gene has indicated the presence of a second amino-terminal domain located between residues 130 and 169 that is required for morphological cell transformation (4, 40; V. W. Wilkerson and J. T. Parsons, manuscript in preparation). A variety of experimental evidence has indicated that the carboxy-terminal half of pp60^{src} contains the functional domain for protein kinase activity (5, 16, 39, 52, 53). The extensive amino acid sequence homology between the carboxy-terminal half of pp60^{src} and other viral tyrosine protein kinases further supports the concept that this portion of pp60^{src} contains the functional catalytic domain or domains necessary for tyrosine protein kinase activity (1). Recently, we showed that mutants containing single amino acid changes within a region that is conserved among virtually all of the tyrosine kinases (the A-P-E or ala$_{430}$-pro$_{431}$-glu$_{432}$ sequence) are defective for cell transformation (5, 40). The biological and biochemical properties of these mutants strengthen the argument that this region of the *src* protein is part of a functional domain whose structural integrity is required for cellular transformation. Recently, Takeya and Hanafusa (56) determined the nucleotide sequence of the coding region of the normal cellular homolog (c-*src*) of the v-*src* gene, pp60$^{c\text{-}src}$, and compared it with the sequence of the v-*src* gene. The deduced amino acid sequences (56) exhibit virtual homology except within the carboxy terminus, where the carboxy-terminal 19 amino acids of pp60$^{c\text{-}src}$ are replaced by a new set of 12 amino acids of pp60$^{v\text{-}src}$. Recently, several groups have shown that overexpression of the c-*src* gene product does not lead to overt cellular transformation (29, 39, 49, 50), indicating that the structural differences between the c-*src* and v-*src* gene products are essential for mediating cellular transformation.

We investigated the role of the carboxy-terminal amino acid sequences in the maintenance of functional *src* protein activity by introducing deletions and oligonucleotide inser-

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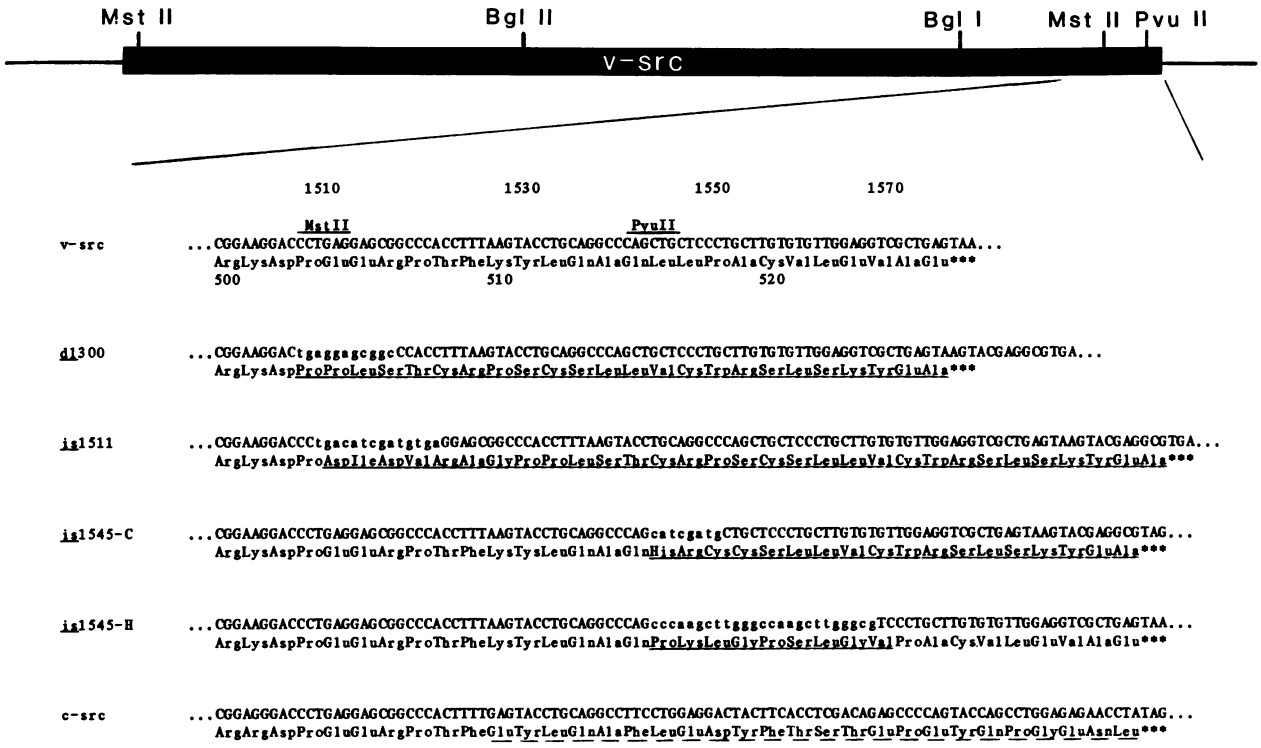

FIG. 1. Nucleotide sequence of mutations within the 3′ terminus of the RSV *src* gene. Insertions of synthetic oligonucleotides are designated by lowercase letters. Amino acid sequences that differ from wild-type viral *src* are underlined. Amino acid sequence differences between v-*src* and c-*src* are indicated with a dashed line. Sequence alterations were identified by digestion with restriction endonuclease and by direct DNA sequence analysis (37). Relevant restriction endonuclease recognition sites within the *src* gene are included.

tions into the 3′ end of the *src* gene. Mutant viruses generated by the transfection of mutagenized viral DNA into chicken cells encode *src* proteins containing alterations in the carboxy-terminal 23 amino acids of pp60^{src}. Analysis of the proteins encoded by these viruses showed that gross structural alterations of the carboxy terminus of pp60^{src} resulted in transformation-defective viruses that encode enzymatically inactive *src* proteins. However, the substitution of sequences from the 3′ end of the c-*src* gene does not lead to an appreciable loss of transforming activity in the hybrid virus, and the *src* protein encoded by these chimeric genes retains wild-type levels of tyrosine protein kinase activity.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Chicken embryo cell cultures were prepared from gs-negative embryos (Spafas) and maintained as previously described (23). Prague A (PrA) RSV DNA (4, 26) used for mutagenesis was contained in a nonpermuted molecular clone of RSV inserted into a pBR322 plasmid vector (pJD100). The transfection of chicken cells with pJD100 or mutagenized pJD100 DNA was carried out by applying 1 to 2 μg of purified plasmid DNA (28) to cultures of chicken cells as described previously (26). Since pJD100 and its mutagenized derivatives contain nonpermuted copies of the RSV genome, transfected cells produce high levels of mutant virus, which was routinely used for subsequent experiments.

Restriction enzyme digestion and agarose gel electrophoresis. Restriction enzymes were purchased from New England Biolabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as recommended by the supplier. DNA restriction fragments were purified either from agarose gels containing low-gelling-temperature agarose (SeaPlaque FMC Corp., Marine Colloids Div., Rockland, Maine) or by adsorption to and elution from DEAE-membrane paper (Schleicher and Schuell, Inc., Keene, N.H.). Southern blot analysis (54) of cellular proviral DNA was carried out as described previously (23).

Construction of mutations. Deletion mutations were introduced at each of the two *Mst*II restriction sites in the *src* gene of the RSV genome (nucleotide positions 25 and 1509) (46) by the partial digestion of pJD100 DNA with *Mst*II, the purification of linear molecules, and treatment with the exonuclease *BAL* 31 (Bethesda Research Laboratories) as described previously (4). After *BAL* 31 digestion, staggered ends were repaired with *Escherichia coli* DNA polymerase Klenow fragment (New England Biolabs) and the linear DNA molecules were ligated with T4 DNA ligase and used to transform *E. coli* HB101 cells (18). The resulting colonies were screened for deletion mutations in the *src* gene by isolating plasmid DNA from 1-ml cultures (28), digesting with *Mst*II, and analyzing the DNA fragments by agarose gel electrophoresis. Insertion mutations were constructed by partially digesting pJD100 DNA with either *Mst*II or *Pvu*II, purifying linear DNA, and religating in the presence of an 8-base-pair (bp) *Cla*I linker or a 12-bp *Hin*dIII linker (New England Biolabs). In the case of DNA digested with *Mst*II, the staggered ends were repaired with Klenow fragment before ligation. Plasmid DNA was used to transform *E. coli* HB101 cells, and the resulting colonies were screened for the

absence of either a *Mst*II or a *Pvu*II restriction site and the acquisition of either a *Cla*I or a *Hin*dIII restriction site. All mutations were confirmed by direct DNA sequencing (37).

Immunoprecipitation and polyacrylamide gel analysis of labeled cell proteins. Infected chicken cells were labeled with [^{35}S]methionine or $^{32}P_i$ for 4 to 6 h as described previously (4, 41, 43). Immunoprecipitation of *src* protein from labeled cell extracts was carried out as follows. Cells were washed twice with STE (0.15 M NaCl, 50 mM Tris hydrochloride [pH 7.2], 1 mM EDTA) and lysed in 2 ml of 10 mM Tris hydrochloride (pH 7.2)–0.1 M NaCl–1 mM EDTA– 0.5% deoxycholate–1% Nonidet P-40. Lysates were clarified at 100,000 × *g* for 30 min and incubated with rabbit anti-p60^{src} serum (22) or rabbit anti-36K serum (19) at 0°C for 60 min. Immune complexes were adsorbed to Formalin-fixed *Staphylococcus aureus* (30) and washed once in lysis buffer, once in 10 mM Tris hydrochloride (pH 7.2)–1 M NaCl–1% deoxycholate–1% Triton X-100–0.1% sodium dodecyl sulfate–1 M urea, and once in the original lysis buffer. The immune complexes were then suspended in sample buffer, boiled, and electrophoresed on a 9.0% polyacrylamide gel (33). Gels were fixed, stained, dried, and autoradiographed. Gels containing ^{35}S-labeled proteins were treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.) to provide fluorographic enhancement before drying. To quantitate the relative amount of pp60^{src} or structurally altered forms of *src* protein in the labeling experiments, we immunoprecipitated equal amounts of labeled protein extract. Immune complex protein kinase assays were performed as described previously (4, 42). To compare the levels of kinase activity of wild-type and structurally altered *src* proteins, we immunoprecipitated parallel cultures of unlabeled and [^{35}S]methionine-labeled cells with antiserum, and determined the amount of pp60^{src} autophosphorylation relative to the level of ^{35}S-labeled *src* protein in immune complexes.

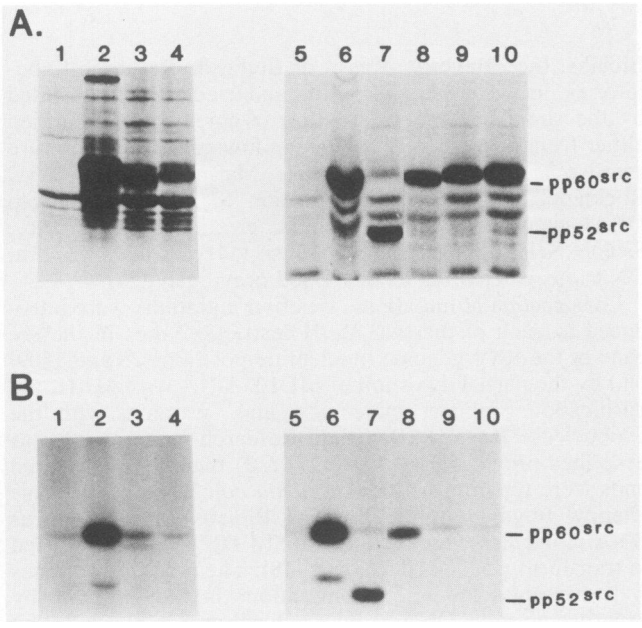

FIG. 2. Immunoprecipitation of *src* protein from cells labeled with [^{35}S]methionine (A) or with $^{32}P_i$ (B). Chicken cells infected with PrA RSV or mutant viruses were labeled with [^{35}S]methionine or $^{32}P_i$, harvested, immunoprecipitated with rabbit anti-p60^{src} serum, and analyzed by polyacrylamide gel electrophoresis and autoradiography as described in the text. Lanes: 1, uninfected cells; 2, PrA-RSV-infected cells; 3, *dl*300-infected cells; 4, *is*1511-infected cells; 5, uninfected cells; 6, PrA-RSV-infected cells; 7, *dl*121-infected cells; 8, *pm*9-infected cells; 9, *is*1545-C-infected cells; 10, *is*1545-H-infected cells.

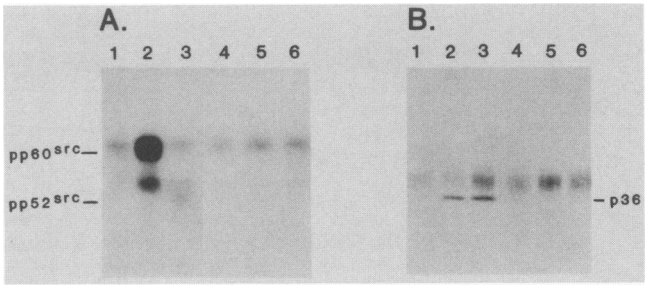

FIG. 3. In vitro and in vivo protein kinase activity of *src* protein from cells infected with PrA RSV or mutant viruses. Parallel cultures of cells were either harvested, immunoprecipitated with rabbit anti-p60^{src} serum, and assayed for autophosphorylation as described in the text (Panel A) or labeled with $^{32}P_i$, harvested, immunoprecipitated with anti-36K-protein serum, and analyzed by polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, uninfected cells; 2, PrA-RSV-infected cells; 3, *dl*121-infected cells; 4, *pm*9-infected cells; 5, *is*1545-C-infected cells; *is*1545-H-infected cells.

Indirect immunofluorescence staining of cells. RSV-infected and uninfected chicken cells were seeded on cover slips and allowed to grow overnight. Cells were fixed in 3% paraformaldehyde, permeabilized in 0.4% Triton X-100 (35), and incubated with 100 μl of anti-*src* monoclonal antibody EB_7 (42) for 30 min at room temperature. The binding of EB_7 antibody was localized by incubation of the cells with goat $F(ab')_2$ anti-mouse immunoglobulin G (Jackson Immuno Research) (10 μg/ml) for 30 min followed by incubation with fluorescein-conjugated rabbit $F(ab')_2$ anti-goat $F(ab')_2$ (Jackson Immuno Research) (10 μg/ml) for an additional 30 min. Between incubations with antibody, the cover slips were washed three times with phosphate-buffered saline. The stained cells were examined with a Leitz fluorescence microscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.) equipped with epi-illumination.

RESULTS

Isolation of 3′ *src* mutations. In our initial experiment, we sought to probe the effects of structural modifications within the carboxy-terminal region of the *src* protein. Therefore, deletion mutations near the 3′ end of the *src* gene were constructed by utilizing a *Mst*II restriction site located 71 bp upstream from the 3′ termination codon of the *src* gene (Fig. 1). Deletions were introduced by exonuclease *BAL* 31 digestion, repair, and religation with T_4 ligase as described above. DNA from individual transformants was digested with *Mst*II, and plasmids containing deletions of the *Mst*II site in the 3′ end of the *src* gene were identified by agarose gel electrophoresis. DNA sequence analysis of one such mutation, *dl*300, revealed a deletion of bp 1509 to bp 1518 in the *src* gene resulting in a deletion of Glu_{504} to Arg_{506} followed by a shift to an alternate reading frame (Fig. 1). When chicken cells were transfected with *dl*300 DNA, no morphological transformation was observed. Two additional mutations within the carboxy-terminal sequences were constructed by linker insertion. A mutation was obtained by the insertion of an eight-bp *Cla*I linker at the same *Mst*II site utilized above (Fig. 1). This mutation, *is*1511, has an inser-

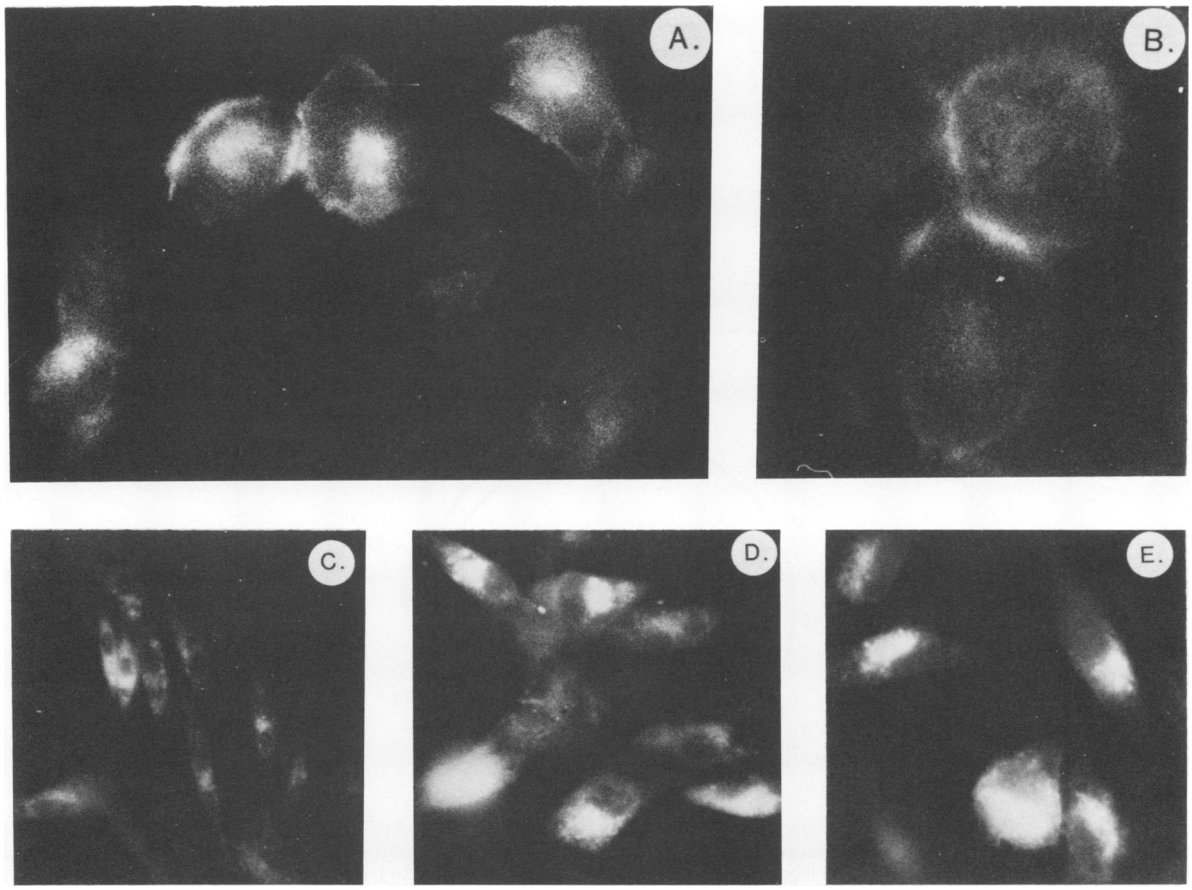

FIG. 4. Immunofluorescent staining of cells infected with PrA RSV or mutant viruses. Cells were grown on cover slips, fixed, permeabilized, and stained with a monoclonal antibody to pp60^{src} (EB_7) as described in the text. (A) and (B), PrA-RSV-infected cells; (C) and (D), *pm*9-infected cells; (E), *is*1545-H-infected cells.

tion of 11 nucleotides (3 nucleotides from the repair of the *Mst*II staggered ends), which resulted in a frameshift altering the last 23 amino acids of pp60^{src}. The insertion of an 8-bp *Cla*I linker at a *Pvu*II restriction site 36 bp from the 3′ end of the *src* gene (Fig. 1) yielded the mutation *is*1545-C, which contains a shift to the same reading frame as the *is*1511 mutation but results in an alteration of only the last 11 amino acids of pp60^{src}. Transfection with DNAs from *is*1511 and *is*1545-C did not induce a morphological transformation of chicken cells. Finally, a 12-bp *Hin*dIII linker was inserted at the 3′ terminal *Pvu*II restriction site, yielding an inframe insertion of 24 nucleotides (the result of the insertion of two linker molecules). This mutation, *is*1545-H, results in an inframe insertion of eight amino acids (Fig. 1). Transfection with DNA from CH*is*1545-H also did not result in a morphological transformation of chicken cells.

Characterization of structurally altered *src* proteins. The expression of the mutant viruses encoding proteins having an altered carboxy terminus was examined in chicken cells infected with high-titered stocks of the mutant viruses CH*dl*300, CH*is*1511, CH*is*1545-C, and CH*is*1545-H. In addition, cells were also infected with two other transformation-defective viruses, CH*dl*121 and CH*pm*9. CH*dl*121 encodes a *src* protein containing a deletion of sequences gly_{82} to arg_{169} (40), and CH*pm*9 contains a single point mutation resulting in a proline-to-serine alteration at residue 431 (6). Infected cells were labeled with [^{35}S]methionine (Fig. 2A) or $^{32}P_i$ (Fig. 2B), labeled cell extracts were immunoprecipitated with rabbit anti-p60^{src} serum, and the immune complexes were analyzed by polyacrylamide gel electrophoresis. Labeling with [^{35}S]methionine revealed that each of the carboxy-terminal mutants encoded an approximately 60,000-molecular-weight (60K) protein, as expected from the nucleotide sequence analysis (Fig. 2A, lanes 3, 4, 9, and 10). However, additional, slightly smaller, *src*-related proteins (molecular weight, 57,000 to 59,000) were often detected in immunoprecipitates of cells infected with *dl*300 and *is*1511 (Fig. 2A, lanes 3 and 4). These lower-molecular-weight proteins appear to be degradation products of pp60^{src}, based on *S. aureus* V8 partial peptide mapping (data not shown). Labeling of parallel cultures with $^{32}P_i$ and immunoprecipitation with anti-p60^{src} serum revealed that the *src* proteins containing carboxy-terminal alterations were significantly underphosphorylated compared with wild-type pp60^{src} (Fig. 2B, lanes 2 and 6), pm9 pp60^{src} (lane 8), or pp52^{src} encoded by the deletion mutant *dl*121 (lane 7). We have also observed that [^{35}S]methionine-labeled immunoprecipitates from cells infected with virus containing carboxy-terminal *src* mutations exhibited diminished amounts of the cellular protein pp90 (D. L. Bryant, V. W. Wilkerson, and J. T. Parsons, unpublished results; L. A. Lipsich and J. Brugge, unpublished results). Since pp90 and a second cellular protein, pp50, are normally associated with soluble pp60^{src} in RSV-transformed cells (2, 38; J. Brugge, Curr. Top. Microbiol. Immunol., in press), it appears that the *src* proteins containing carboxy-terminal alterations do not associate with pp90

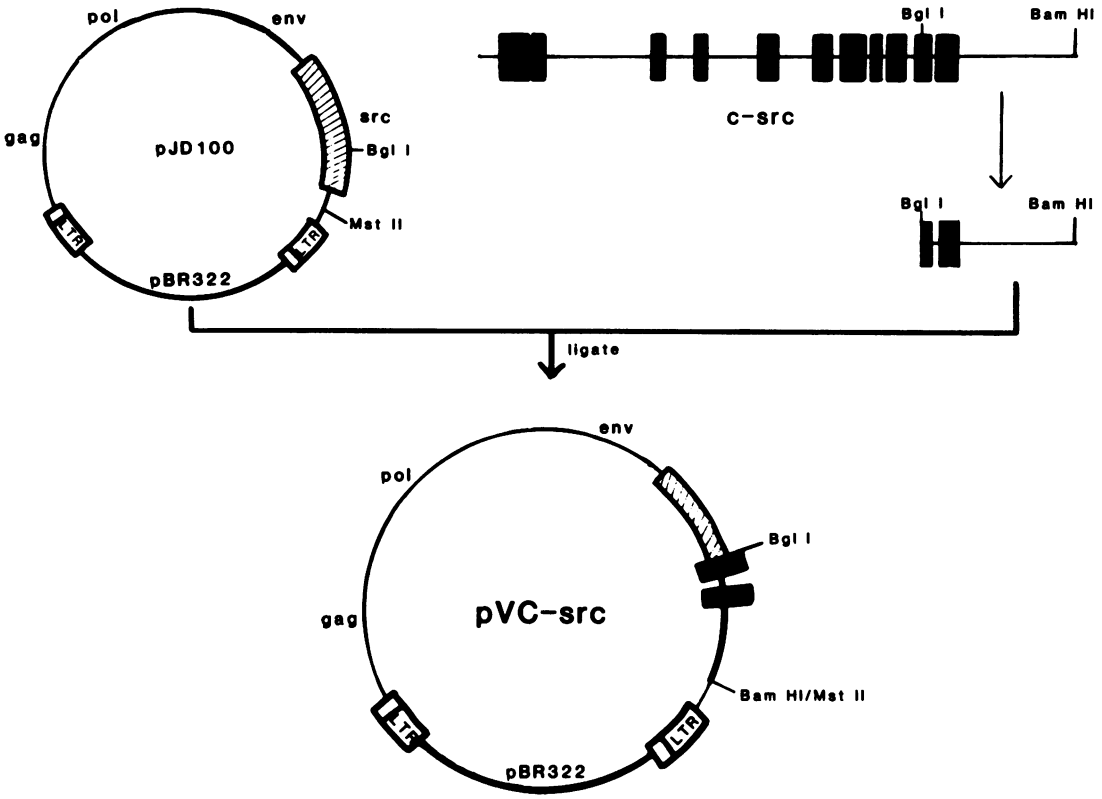

FIG. 5. Construction of the plasmid pVC*src* containing a chimeric *src* gene. The plasmid pJD100 was digested with *Mst*II, the ends were repaired with Klenow fragment of DNA polymerase I, and then the plasmid was digested with *Bgl*I yielding a vector fragment missing the 3′ end of the viral *src* gene. The cognate c-*src* fragment was prepared from the plasmid pSVOHCS11 by digestion with *Bam*HI and repaired with Klenow fragment as described previously. Digestion with *Bgl*I, which cleaves the c-*src* DNA at the identical site as v-*src*, yielded a *Bgl*I-blunted *Bam*HI c-*src* fragment, which was then inserted into the *Bgl*I-blunted *Mst*II pJD100 vector to form the chimeric plasmid pVC*src*.

and pp50 or that the complex is unstable in mutant-infected cells. The lack of association of pp90 and pp50 with the *src* protein encoded by *is*1545 has been confirmed by immunoprecipitation with antiserum to the pp90 protein (Lipsich and Brugge, personal communication).

To determine the effects of alterations of the carboxy terminus on tyrosine protein kinase activity, we infected cells with mutant or wild-type RSV and immunoprecipitated the lysates with anti-p60^{src} serum; we also incubated the immune complexes with γ-[^{32}P]ATP to measure the level of *src* autophosphorylation. The 3′ mutations *is*1545-C and *is*1545-H encode *src* proteins with significantly reduced levels of in vitro phosphorylation activity (Fig. 3A, lanes 5 and 6). The level of kinase activity observed was similar to that exhibited by immune complexes from cells infected with the point mutation pm9 (lane 4). Immune complexes from cells infected with *dl*121, an amino-terminal deletion mutation, also exhibited a level of tyrosine kinase activity reduced from wild-type levels (lane 3). The phosphorylation of the cellular 36K protein, which is phosphorylated predominantly on a tyrosine residue in RSV-infected cells (19, 46), was also examined in mutant-infected cells (Fig. 3B). Parallel cultures of infected cells were labeled with $^{32}P_i$ and [^{35}S]methionine, and extracts were prepared and immunoprecipitated with anti-36K-protein serum. In cells infected with virus bearing the carboxy-terminal *src* mutation, the 36K protein was underphosphorylated compared with *dl*121-infected or wild-type RSV-infected cells (Fig. 3B). Similar levels of 36K protein were present in each cell lysate as determined by the immunoprecipitation of [^{35}S]methionine-labeled cells (data not shown). These results reveal that structural perturbation of the carboxy-terminal sequence, as delineated by the mutations shown in Fig. 1, results in RSV mutants defective for cell transformation. Such mutants encode structurally altered *src* proteins significantly diminished in their capacity to phosphorylate substrates both in vitro and in vivo.

Intracellular localization of structurally altered *src* proteins. Wild-type pp60^{src} in RSV-infected cells is primarily located in the plasma membrane (14, 32). In addition, there is evidence suggesting that the association of pp60^{src} with pp50 and pp90 is involved in the transport of pp60^{src} to the plasma membrane (14; J. Brugge, in press). We examined the intracellular distribution of the altered *src* proteins by immunofluorescence localization techniques (35). Cells infected with PrA RSV, CH*pm*9 (6), and CH*is*1545-C were grown on cover slips, fixed with formaldehyde, permeabilized, and stained with monoclonal antibody to pp60^{src}. Cells infected with either CH*pm*9 (Fig. 4C and D) or CH*is*1545-C (Fig. 4E) routinely exhibited an asymmetric perinuclear pattern of fluorescence with no apparent localization of *src* protein at the plasma membrane or within adhesion plaques or cell-cell junctions. In contrast, wild-type virus-infected cells exhibited staining at the plasma membrane and cell-cell junctures (Fig. 4A and B). The altered pattern of pp60^{src} distribution within the cell, as revealed by the fluorescence localization experiments, suggests that structural modifications resident in the *src* proteins encoded by CH*is*1545-C or

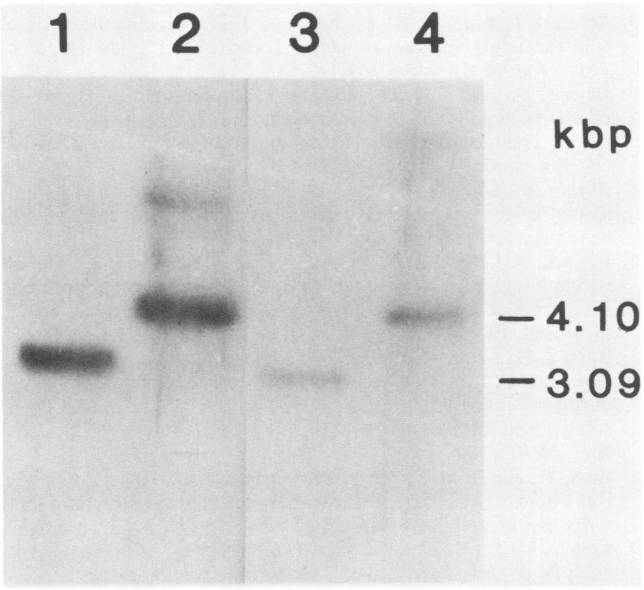

FIG. 6. Southern blot analysis of chicken cells infected with PrA RSV or pVC*src*. Total cell DNA was isolated from cells infected with PrA RSV or pVC*src*, digested with *Eco*RI, electrophoresed on an agarose gel, and transferred to nitrocellulose by the method of Southern (54). A ^{32}P-labeled 1.7-kilobase *Mst*II fragment from pJD100 containing the entire v-*src* gene was used to probe the cell DNA blot for *src*-specific fragments. Lanes: 1, pJD100 DNA; 2, pVC*src* DNA; 3, PrA-RSV-infected cell DNA; 4, pVC*src*-infected cell DNA.

CH*pm*9 influence the localization of *src* to the plasma membrane. Preliminary biochemical analysis of the localization of the *src* protein encoded by CH*is*1545-C revealed a less stable association with cellular membrane compared with wild-type pp60^{src} (Lipsich and Brugge, unpublished).

Construction and analysis of a chimeric *src* gene bearing the 3′ end of the c-*src* gene. Direct DNA sequencing of the v-*src* and c-*src* genes has shown that the carboxy-terminal 19 amino acids of pp60$^{c\text{-}src}$ were replaced by a new sequence of 12 amino acids of pp60$^{v\text{-}src}$ (Fig. 1). Because of our observations that mutations introduced into the v-*src* gene proximal to the site of sequence divergence between v-*src* and c-*src* (Fig. 1) result in a substantial reduction in transforming activity and protein kinase activity, we undertook to construct a hybrid *src* gene containing nucleotide sequences derived from v-*src* and c-*src* genes. The hybrid *src* gene (v-c-*src*) (Fig. 5) encodes an *src* protein containing amino acid residues 1 to 431 of v-*src* and residues 432 to 533 of c-*src*. The transfection of chicken cells with the plasmid DNA containing the hybrid *src* gene resulted in cellular transformation; however, foci were detected 7 to 10 days later than in cells transfected with plasmid DNA containing only the wild-type viral DNA. Because of the delay in the appearance of transformed foci in cells transfected with the hybrid *src* gene, the structure of the integrated proviral DNA was examined to determine if rearrangement of the *src* gene had occurred during the infection process. Figure 6 shows a Southern blot of DNA isolated from cells transfected with either wild-type PrA DNA (pJD100) or the hybrid v-c-*src* DNA (pVC*src*) and digested with the restriction enzyme *Eco*RI. Cells transformed with wild-type DNA contained the characteristic 3.09-kilodalton *Eco*RI *src* fragment (23), whereas cells transfected with pVC*src* DNA contained a 4.2-kilodalton *Eco*RI *src* fragment (Fig. 6). Immunoprecipitation of cells infected with pVC*src* and labeled with [^{35}S]methionine revealed the presence of a 61-kilodalton protein whose electrophoretic mobility was slightly slower than pp60$^{v\text{-}src}$ (Fig. 7A). The change in the electrophoretic mobility of pp60$^{v\text{-}c\text{-}src}$ is consistent with the predicted sequence alteration of the c-*src* protein. In a parallel experiment, the protein kinase activity of pp61$^{v\text{-}c\text{-}src}$ was compared with that of pp60$^{v\text{-}src}$. Both *src* proteins exhibited similar activities measured by either the *src* autophosphorylation (Fig. 7B) or the phosphorylation of the exogenous substrate casein (data not shown). These data clearly indicate that replacement of the carboxy-terminal sequences of v-*src* with the cognate sequences of c-*src* does not significantly alter the ability of the *src* gene product to mediate cellular transformation or to function in vitro as a tyrosine protein kinase.

DISCUSSION

We used site-directed mutagenesis techniques to isolate deletion mutations and linker insertion mutations within the 3′ end of the RSV *src* gene. Analysis of a deletion mutation, insertion-frameshift mutations, and an inframe insertion mutation revealed that structural alteration of the carboxy-terminal portion of the pp60^{src} protein leads to the loss of cell-transforming function and a loss in tyrosine protein kinase activity. The minimal mutation required to inactivate the *src* protein in this study was an insertion of nine amino acids at position 415 of the *src* protein sequence. Interestingly, this insertion mutation occurred adjacent to the point of sequence divergence between the v-*src* and c-*src* proteins. However, when a chimeric v-c-*src* gene was constructed and introduced into chicken cells in the context of an RSV vector, we observed transformation of the cells and identified an enzymatically active hybrid *src* protein by immunoprecipitation of cell extracts. The precise nature of the structural motif that was altered by our mutations is unclear at this time; however, a comparison of the deduced amino acid sequences of the tyrosine kinases encoded by the *fps* and *yes* oncogenes reveals a highly conserved leucine residue at position 517 (31, 51). This residue was present in pp60$^{c\text{-}/5src}$ but was altered (replaced) in the mutant *is*1545-H (Fig. 1). It is possible that this residue is critical in the proper

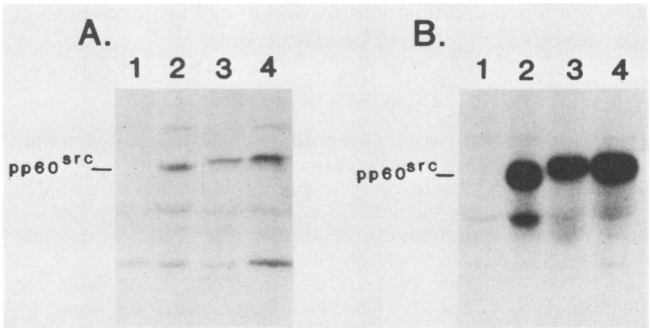

FIG. 7. Identification of *src* proteins in cells infected with virus containing a v-c-*src* gene. Parallel cultures of chicken cells were either labeled with [^{35}S]methionine, harvested, immunoprecipitated with rabbit anti-p60^{src} serum, and analyzed by polyacrylamide gel electrophoresis (A) or harvested, immunoprecipitated with anti-p60^{src} serum, and assayed for autophosphorylation as described in Fig. 2. Lanes: 1, uninfected cells; 2, PrA-RSV-infected cells; 3, pVC*src* (clone 2)-infected cells; 4, pVC*src* (clone 15)-infected cells. Clones 2 and 15 of pVC*src* represent independent isolates of the plasmids bearing the chimeric v-c-*src* gene.

folding of the carboxy-terminal portion of the *src* protein sequence.

src proteins having a structurally altered carboxy terminus exhibited a significant reduction in tyrosine protein kinase activity both in vitro, by the functional immune complex kinase assay, and in vivo, by the degree of tyrosine phosphorylation of the 36K protein, a protein readily phosphorylated on tyrosine in vivo in RSV-transformed cells. That the underphosphorylation of the 36K protein is a consequence of a mutation within the catalytic domain giving rise to inactive mutant *src* protein is supported by the observation that cells infected with the amino-terminal deletion mutant CH*dl*121 contain significant levels of phosphorylated 36K protein. It is interesting to note that, as we reported earlier with mutations within the A-P-E-A sequence of pp60^{src} (6), *src* proteins which contain an altered carboxy terminus and have reduced tyrosine protein kinase activity are also underphosphorylated (Fig. 1B) on both serine and tyrosine (Bryant, unpublished observations).

Our observations that the alteration of the carboxy terminus of pp60$^{v\text{-}src}$ has a deleterious effect on cell transformation as well as protein kinase activity suggests that the carboxy-terminal portion of pp60$^{v\text{-}src}$ is involved in maintaining the overall tertiary structure of the enzymatically active *src* protein. Gentry et al. (21) have observed that an antibody to a pentadecapeptide corresponding in sequence to residues 498 to 512 of the *src* protein inhibits pp60^{src} protein kinase activity. These data are consistent with the view that the carboxy-terminal portion of the *src* protein is important in maintaining the structure of a functional catalytic domain. We have considered the question of whether the structural changes manifest in pp60$^{c\text{-}src}$ (56) alter the functional activity of the c-*src* protein. In agreement with Iba et al. (29), we found that virus containing a chimeric *src* gene encoding residues 1 to 431 of v-*src* and residues 432 to 533 of c-*src* induced the transformation of chicken cells, albeit more slowly than did wild-type RSV. To date, we have been unable to demonstrate any clear biological or biochemical differences between the *src* proteins encoded by the hybrid and wild-type *src* genes. Our observations appear to differ from those of Shalloway et al. (49), who reported that a similar hybrid *src* gene was inefficient in the transformation of mouse 3T3 cells. Transfection of mouse 3T3 cells with pVC*src* DNA has consistently yielded foci of transformed cells, but a detailed characterization of cell lines established from such foci remains to be carried out.

ACKNOWLEDGMENTS

We thank Betty Creasy, Amy Coffman, and Renee Renaud for excellent technical assistance and Jon Morgan and Mark Heaney for many helpful discussions. We are particularly indebted to Sarah Parsons for providing the antiserum to pp60^{src} and to Ray Erikson for providing the antibody to the 36K protein. The c-*src* plasmid pSVOHCS11 was generously provided by Tona Gilmer.

J.T.P. is a recipient of a Faculty Research Award from the American Cancer Society. This investigation was supported by Public Health Service grants CA29243 and CA27578 awarded by the National Cancer Institute and grant NP-462G from the American Cancer Society.

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